Mammalian target of rapamycin regulates miRNA-1 and follistatin in skeletal myogenesis

Yuting Sun,1 Yejing Ge,1 Jenny Drnevich,2 Yong Zhao,3,4 Mark Band,2 and Jie Chen1

1Department of Cell and Developmental Biology and 2W.M. Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign, Champaign, IL 61820
2Center for Molecular Cardiology and 3Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York, NY 10029

Mammalian target of rapamycin (mTOR) has emerged as a key regulator of skeletal muscle development by governing distinct stages of myogenesis, but the molecular pathways downstream of mTOR are not fully understood. In this study, we report that expression of the muscle-specific micro-RNA (miRNA) miR-1 is regulated by mTOR both in differentiating myoblasts and in mouse regenerating skeletal muscle. We have found that mTOR controls MyoD-dependent transcription of miR-1 through its upstream enhancer, most likely by regulating MyoD protein stability. Moreover, a functional pathway downstream of mTOR and miR-1 is delineated, in which miR-1 suppression of histone deacetylase 4 (HDAC4) results in production of follistatin and subsequent myocyte fusion. Collective evidence strongly suggests that follistatin is the long-sought mTOR-regulated fusion factor. In summary, our findings unravel for the first time a link between mTOR and miRNA biogenesis and identify an mTOR–miR-1–HDAC4–follistatin pathway that regulates myocyte fusion during myoblast differentiation in vitro and skeletal muscle regeneration in vivo.

Introduction

Skeletal myoblast fusion, which results in the formation of multinucleated myofibers, is critical for embryonic muscle development, adult muscle regeneration and maintenance, and muscle hypertrophy under certain conditions. The molecular mechanisms underlying myoblast fusion represent one of the central questions in skeletal muscle biology (Wakelam, 1985; Jansen and Pavlath, 2008). Two molecularly separable stages of fusion have been identified in mammalian muscle cells (Jansen and Pavlath, 2008). After an early stage of differentiation, including cell cycle withdrawal, myogenin expression, and contractile protein expression, mononucleated myoblasts fuse to form nascent myofibers/myotubes. Subsequently, growth and maturation of the muscle cells are achieved through a second-stage fusion, which occurs between the nascent myofibers/myotubes and myoblasts. Although many regulators of these fusion processes have been revealed in recent years (Jansen and Pavlath, 2008), a better understanding of the regulation is still needed.

Y. Sun and Y. Ge contributed equally to this paper.

Correspondence to Jie Chen: jiechen@illinois.edu

Abbreviations used in this paper: AI, after injury; HDAC, histone deacetylase; IGF, insulin-like growth factor; KI, kinase inactive; LNA, locked nucleic acid; MHC, myosin heavy chain; miRNA, micro-RNA; mTOR, mammalian target of rapamycin; qRT-PCR, quantitative RT-PCR; RR, rapamycin resistant; shRNA, small hairpin RNA; TA, tibialis anterior; TSA, trichostatin A.

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Despite the well-recognized importance of both miRNAs and mTOR in myogenesis, a possible connection between the two has never been implicated or examined. In this study, we present evidence revealing for the first time the regulation of a miRNA by mTORC1 signaling. Furthermore, we have identified follistatin as the fusion factor regulated by mTORC1 signaling through miR-1 and histone deacetylase 4 (HDAC4) in myoblast differentiation and muscle regeneration.

Results
mTORC1 regulates miR-1 levels in skeletal muscle

To examine a potential link between miRNA and mTORC1 signaling in skeletal myogenesis, we performed miRNA microarray analyses. We compared miRNA expression profiles of C2C12 cells at differentiation time 0 and 72 h, the latter with or without rapamycin treatment. Among the miRNAs up-regulated during differentiation (Fig. S1 and Table S1), miR-1 expression was drastically inhibited by rapamycin. Quantitative RT-PCR (qRT-PCR) results confirmed that miR-1 increased dramatically during C2C12 differentiation (Fig. 1 A), which is consistent with a previous study (Chen et al., 2006), and the increase was almost completely blocked by rapamycin treatment. As expected, rapamycin abolished myotube formation (not depicted) and drastically inhibited the expression of myogenic markers, including myogenin, myosin heavy chain (MHC), MEF2A, and MEF2C (Fig. S2). To directly confirm mTOR’s role in controlling miR-1 levels, we knocked down mTOR in C2C12 cells ex vivo (Hornberger et al., 2006). The regulation of skeletal myocyte differentiation by mTORC1 occurs at two stages via distinct mechanisms. mTORC1 controls the initiation of myoblast differentiation by regulating IGF-II expression (Erbay and Chen, 2001; Erbay et al., 2003), whereas a late-stage myocyte fusion leading to myotube maturation is regulated by mTORC1 through a yet to be identified secreted factor (Park and Chen, 2005). These regulatory mechanisms are also recapitulated by mTOR functions in muscle regeneration in vivo (Ge et al., 2009).

Micro-RNAs (miRNAs) are a class of small noncoding RNAs that regulate protein expression mainly by targeting the 3' untranslated region of messenger RNAs (Bartel, 2004, 2009). Intensive studies in recent years have revealed miRNA as a principal regulatory mechanism of gene expression, governing numerous biological processes across the species (Bushati and Cohen, 2007; Bartel, 2009). Several miRNAs have been recognized as important modulators in the development of skeletal and cardiac muscle (van Rooij et al., 2008). Among them, miR-1 is a conserved muscle-specific miRNA that is essential for myogenesis. In Caenorhabditis elegans, miR-1 regulates synaptic functions at the neuromuscular junctions (Simon et al., 2008). In Drosophila melanogaster, deletion of miR-1 leads to defects in muscle differentiation or maintenance, presumably by removing the inhibition on the Notch ligand Delta (Sokol and Ambros, 2005). In mammals, deletion of miR-1-2 in mice causes dysregulation of cardiogenesis (Zhao et al., 2005, 2007), and inhibiting miR-1 impairs the differentiation of skeletal myoblasts (Chen et al., 2006). Despite the well-recognized importance of both miRNAs and mTOR in myogenesis, a possible connection between the two has never been implicated or examined. In this study, we present evidence revealing for the first time the regulation of a miRNA by mTORC1 signaling. Furthermore, we have identified follistatin as the fusion factor regulated by mTORC1 signaling through miR-1 and histone deacetylase 4 (HDAC4) in myoblast differentiation and muscle regeneration.

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which showed accumulation of miR-1 signals adjacent to both centrally and peripherally localized nuclei (Fig. S3, enlarged image) and dramatic decrease of signals upon rapamycin administration. Collectively, our observations suggest that mTORC1 controls the levels of miR-1 in skeletal myocytes both in vitro and in vivo.

mTORC1 regulates transcription of miR-1 through an upstream enhancer

miRNAs are transcribed as pri-miRNAs, cleaved by Drosha to form pre-miRNAs, followed by another cleavage by Dicer, which results in mature miRNAs. A blockage by rapamycin at any of those steps would result in a decrease of mature miR-1 levels. As revealed by Northern blotting (Fig. 2 A), rapamycin treatment of differentiating C2C12 cells drastically decreased the level of mature miR-1 without inducing pre-miR-1 (73 and 78 nucleotides), indicating that rapamycin is unlikely to block the processing of pre-miR-1 to miR-1. The lack of detectable pre-miR-1 by Northern analysis is consistent with rapid processing by Dicer observed for most miRNAs. To test whether the decrease of mature miR-1 could be attributed to a blockage in the processing of pri-miR-1 to pre-miR-1, we measured pri-miR-1 levels by qRT-PCR. MiR-1 is encoded at two chromosomal loci, resulting in two distinct primary transcripts. As shown in Fig. 2 B, rapamycin inhibited rather than enhanced the levels of both forms of pri-miR-1, which excludes the possibility that suppression of pri-miR-1 processing is responsible for using lentivirus-delivered small hairpin RNA (shRNA) with two independent target sequences, which inhibited S6K1 phosphorylation and suppressed MHC expression (Fig. 1 B). At the same time, the miR-1 level during differentiation was suppressed by mTOR knockdown (Fig. 1 C).

To validate the dependence of miR-1 levels on mTORC1 in vivo, we examined miR-1 expression in mouse regenerating skeletal muscle. Muscle regeneration was induced upon injury elicited by barium chloride (BaCl₂) injection into the tibialis anterior (TA) muscle (Caldwell et al., 1990; Ge et al., 2009). On various days after injury (AI), the TA muscle was isolated, and miR-1 levels were measured by qRT-PCR. miR-1 was found to increase during regeneration, and the increase was blocked by daily rapamycin administration to the mice (Fig. 1 D), accompanied by inhibition of regeneration as we reported previously (Ge et al., 2009). It should be mentioned that others reported a decrease of miR-1 levels in injured muscles (Yuasa et al., 2008; Greco et al., 2009), which was not observed by us at any time point from day 1 to day 21 AI (Fig. 1 D). It has been proposed that the temporary decrease of miR-1 during muscle injury is associated with the loss of myofibers and induction of fibrosis upon injury, rather than muscle regeneration (Greco et al., 2009). Thus, it is possible that miR-1 levels do not decline in our experimental system, owing to less extensive tissue damage induced by BaCl₂ compared with the methods used by others (Yuasa et al., 2008; Greco et al., 2009). miR-1 expression in regenerating muscles was also confirmed by in situ hybridization, which showed accumulation of miR-1 signals adjacent to both centrally and peripherally localized nuclei (Fig. S3, enlarged image) and dramatic decrease of signals upon rapamycin administration. Collectively, our observations suggest that mTORC1 controls the levels of miR-1 in skeletal myocytes both in vitro and in vivo.
To further investigate the possibility that mTORC1 signaling might regulate the transcription of miR-1, we considered the upstream enhancers for the two miR-1 genes that had been found to regulate the expression of miR-1 in cardiac muscle (Zhao et al., 2005). We set out to examine luciferase reporters for these enhancers in C2C12 cells. As shown in Fig. 3 A, the miR-1-2 enhancer reporter activity increased during C2C12 differentiation, indicating that the enhancer regulating miR-1 in cardiac muscle is also functional in skeletal muscle. Remarkably, rapamycin treatment abolished the increase of the enhancer activity (Fig. 3 A, gray bars). A reporter for the miR-1-1 enhancer displayed similar up-regulation during differentiation and inhibition by rapamycin (unpublished data). To ask whether the effect of rapamycin on miR-1 enhancer was direct or a consequence of rapamycin inhibition of differentiation, we examined the effect of inhibiting phosphatidylinositol-3-kinase and p38, two pathways required for C2C12 differentiation (Kaliman et al., 1996; Cuenda and Cohen, 1999). Strikingly, prolonged (3 d) treatment of C2C12 cells with either wortmannin or SB203580, specific inhibitors of phosphatidylinositol-3-kinase and p38-MAPK, respectively, had no impact on the miR-1 enhancer reporter activity (Fig. 3 B), although both drugs drastically inhibited the differentiation of C2C12 cells from which reporter assays were performed. These observations, together with the fact that not all miRNAs regulated during differentiation were sensitive to rapamycin (Fig. S1 and Table S1), suggest that mTORC1 signaling may directly regulate miR-1 expression through the upstream enhancer.

mTORC1 regulates miR-1 through MyoD

Next, we set out to probe the mechanism underlying mTORC1 regulation of miR-1 transcription. MyoD is a transcription factor that is essential and specific for skeletal myogenesis. Furthermore, putative MyoD-binding sites have been found in both miR-1-1 and miR-1-2 enhancers (Zhao et al., 2005). Indeed, expression of recombinant MyoD in C3H10T1/2 cells, which lack endogenous MyoD expression, drastically stimulated the expression of miR-1 (Fig. 4 A). The expression of recombinant MyoD also markedly activated the miR-1 enhancer reporter in the same cells (Fig. 4 B). A reporter with the putative MyoD-binding site on the enhancer mutated was found to be almost completely insensitive to the expression of MyoD (Fig. 4 B), suggesting that the effect of MyoD on the enhancer is most likely direct, rather than a consequence of MyoD induction of differentiation in C3H10T1/2 cells (Davis et al., 1987).

Consistent with a role of mTORC1 in regulating miR-1 transcription, rapamycin significantly diminished MyoD-induced miR-1 enhancer activity during 24 h of differentiation in C3H10T1/2 cells (Fig. 4 C). Furthermore, we observed that a short period (4 h) of rapamycin treatment was as effective as 24-h treatment in suppressing MyoD-induced miR-1 enhancer activity (Fig. 4 D), once again confirming that the regulation of miR-1 expression by mTORC1 through MyoD is direct. The incomplete rapamycin inhibition of the reporter activity may suggest the existence of additional pathways in the regulation of MyoD activity, but an equally likely possibility is that overexpression of MyoD partially overrides regulation by mTORC1,
rendering MyoD somewhat constitutively active and thus partially resistant to rapamycin.

To gain further insight into the mechanism by which mTOR may control MyoD activity, we examined the endogenous MyoD protein upon rapamycin treatment. As shown in Fig. 4 E, rapamycin treatment induced reduction of MyoD protein levels in C2C12 cells. The rapid response to rapamycin exposure (4 h) is consistent with a direct effect of rapamycin on MyoD levels. In addition, the proteasome inhibitor MG-132 completely reversed the effect of rapamycin on MyoD, suggesting that the stability of MyoD is regulated in a proteasome-dependent manner. A similar effect of rapamycin was found on the level of recombinant MyoD in C3H10T1/2 cells (Fig. 4 F), further confirming that the decrease of MyoD protein is post-translational. Indeed, rapamycin treatment has no effect on the mRNA levels of MyoD in C2C12 cells (Fig. S4 A) or of recombinant MyoD in C3H10T1/2 cells (Fig. S4 B). Collectively, our data suggest that mTORC1 controls MyoD by suppressing its proteasome-dependent degradation.

miR-1 regulates skeletal myogenesis through HDAC4 and follistatin

To delineate the pathway downstream of mTOR and miR-1 in myogenesis, we first considered the targets of miR-1. Several potential targets of miR-1 in skeletal and cardiac muscles have been reported (Zhao et al., 2005, 2007; Chen et al., 2006; Yang et al., 2007). Specifically, miR-1 was shown to promote the differentiation of skeletal myoblast by suppressing the expression of HDAC4, a class II HDAC (Chen et al., 2006). It was also reported that HDAC inhibitors induced myocyte fusion through the production of follistatin (Iezzi et al., 2004), but the identity of the HDAC was not known. We hypothesized that miR-1 might promote skeletal myogenesis by suppressing HDAC4 and subsequently inducing follistatin expression. To examine this hypothesis, we first asked whether HDAC4 could be involved in follistatin production and myogenesis. To this end, we knocked down HDAC4 in C2C12 cells with a lentivirus-delivered shRNA (Fig. 5 A). Depletion of HDAC4 led to an increase in follistatin mRNA levels (Fig. 5 B) as well as the amount of secreted follistatin protein (Fig. 5 C). Meanwhile, HDAC4 knockdown enhanced myocyte differentiation (Fig. 5 D) with an increase in fusion index, defined as the percentage of nuclei in multinucleated cells (Fig. 5 E). These data suggest that HDAC4 suppresses follistatin production and is a negative regulator of myogenic fusion.

Next, we examined the requirement of miR-1 for follistatin expression. Inhibition of miR-1 function was achieved by a locked nucleic acid (LNA)–containing antisense oligonucleotide approach that had been reported to be effective in blocking the actions of the targeted miRNAs (Naguibneva et al., 2006a). As shown in Fig. 6 A, anti–miR-1 significantly suppressed the mRNA levels of follistatin, suggesting that miR-1 is required for the expression of follistatin. In addition, the inhibition of follistatin by anti–miR-1 was almost completely reversed when cells were treated with the HDAC inhibitor trichostatin A (TSA; Fig. 6 A). These data are consistent with a cascade in which miR-1 regulates follistatin expression through impairing an HDAC. At the same time, a negative effect of
anti–miR-1 was found on C2C12 myogenic differentiation (Fig. 6 B), reflected by suppressed expression of myogenin and MHC (Fig. 6 C). The percentage of myotubes containing five or more nuclei was significantly reduced by anti–miR-1 (Fig. 6 D), and the overall fusion index was also lower in anti–miR-1–expressing cells (Fig. 6 E). More importantly, addition of recombinant follistatin to the cell medium fully rescued the myogenic protein expression (Fig. 6 C), myotube size (Fig. 6 D), and fusion index (Fig. 6 E) in the presence of anti–miR-1. Inhibition of HDAC by TSA had a similar rescue effect (Fig. 6, C–E).

We further probed the specific role of HDAC4 in mediating miR-1 regulation of follistatin. We found that anti–miR-1 indeed enhanced the expression of HDAC4 modestly but consistently (Fig. 7 A). Furthermore, knockdown of HDAC4 completely overrode the negative effect of anti–miR-1 on follistatin expression (Fig. 7 B) and, at the same time, restored myocyte fusion as indicated by both the fusion index and the percentage of large myotubes (Fig. 7 C). Collectively, these observations strongly support the existence of a miR-1–HDAC4–follistatin cascade in the regulation of myogenesis.
This outcome was not unexpected, as rapamycin would block the production of both the fusion factor and IGF-II, the latter required for the initiation of differentiation (Erbay et al., 2003). To bypass the initiation of differentiation, thus allowing examination of myocyte fusion specifically, we added recombinant IGF-II to the differentiation medium, which would fully support the initial differentiation and formation of nascent myotubes in the presence of rapamycin (Erbay et al., 2003; Park and Chen, 2005). In the presence of both IGF-II and rapamycin, C2C12 cells differentiated into myotubes but arrested at a small myotube size with fewer myonuclei than mature myotubes (Fig. 9, A and C). Strikingly, the addition of recombinant follistatin in this system led to a complete rescue of mature myotube size (Fig. 9, A and C), suggesting that follistatin is a strong candidate for an mTORC1-regulated second-stage fusion factor.

Previously, we had reported that C2C12 cells stably expressing a rapamycin-resistant (RR) and kinase-inactive (KI) mTOR differentiated in the presence of rapamycin but arrested at the nascent myotube stage (Park and Chen, 2005). To confirm that follistatin was the missing factor in those cells, we added follistatin to the medium of the RR/KI mTOR cells containing rapamycin. Remarkably, this resulted in a dramatic increase of myotube size, as indicated by the higher percentage of myotubes containing five or more nuclei (Fig. 9, B and D). The effectiveness of follistatin was comparable with that of conditioned medium from fully differentiated C2C12 cells. In addition, inhibiting HDAC by TSA had a similar effect (Fig. 9, B and D). Collectively, these data strongly suggest that follistatin is a fusion factor regulated by mTORC1.

**mTORC1 regulates myocyte fusion through follistatin**

Now that we have established a regulatory pathway from miR-1 to follistatin via HDAC4, the immediate question is whether mTORC1 controls this pathway, in other words, whether inhibition of mTORC1 enhances HDAC4 expression and in turn suppresses follistatin expression. Indeed, we found that rapamycin treatment increased HDAC4 protein levels (Fig. 8 A) and at the same time markedly suppressed the mRNA level of follistatin (Fig. 8 B). Notably, rapamycin’s inhibitory effect on follistatin was only obvious at a later stage during differentiation (days 2 and 3) when myocyte fusion takes place, supporting the idea that follistatin is a myogenic factor that promotes fusion rather than the initiation of differentiation. The comparable kinetics of miR-1 and follistatin expression (dramatic increase of both at 48 and 72 h of differentiation; Fig. 1 A and Fig. 8 B) are also consistent with miR-1 regulation of follistatin.

Previously, we demonstrated that mTORC1 regulates at least two distinct processes of myogenesis and that its regulation of the second-stage myocyte fusion leading to myotube maturation or growth is through a yet to be identified secreted factor (Park and Chen, 2005). In light of the discovery of a connection between mTORC1 and follistatin through miR-1 and HDAC4, we wondered whether follistatin might be the long-sought second-stage fusion factor under the control of mTOR. To this end, we tested whether follistatin could rescue myogenic differentiation from rapamycin inhibition. When C2C12 cells were induced to differentiate in the presence of rapamycin, follistatin alone failed to rescue differentiation (unpublished data).
Follistatin is regulated by mTORC1 during muscle regeneration in vivo

Regeneration of damaged adult skeletal muscles involves satellite cell activation and proliferation followed by myoblast differentiation and fusion. Systemic administration of rapamycin impairs the regeneration of skeletal muscle induced by BaCl2 injection in mice, suggesting that mTORC1 plays a key role in muscle regeneration (Ge et al., 2009). Significantly, follistatin mRNA levels increased by about twofold during regeneration, as 1. Data shown are mean ± SD of three independent experiments.

Discussion

Our study has revealed for the first time regulation of an miRNA by mTOR, a master regulator of cell growth and differentiation. We have also identified follistatin as the long-sought myogenic fusion factor under the regulation of mTORC1 signaling (Park and Chen, 2005). We propose a myogenic pathway (Fig. 10 D) in which rapamycin-sensitive mTORC1 controls MyoD-dependent transcription of miR-1, which in turn suppresses HDAC4 and subsequently up-regulates the production of follistatin, stimulating skeletal myocyte fusion in vitro and in vivo. The linearity of this pathway presented in this study would be an oversimplification of the actual regulatory network, as miR-1 and HDAC4 would most likely have multiple targets in myogenesis, and mTOR certainly regulates other myogenic pathways independent of miR-1. Nevertheless, our study identifies a functional pathway important for myocyte fusion and muscle growth, providing a new target for therapeutic intervention in muscle repair and regeneration.

mTOR regulation of miRNA

Best known as a regulator of protein synthesis through its effectors S6K1 and 4E-BP1 (Gingras et al., 2001; Hay and Sonenberg, 2004), the rapamycin-sensitive mTORC1 also regulates mRNA polymerase I–dependent ribosomal DNA transcription through the initiation factors TIF-IA, SL-1, and upstream-binding factor (Mayer and Grummt, 2006). Regulation of mRNA expression has been implicated for mTORC1 as well, although the mechanisms remain to be deciphered. Our discovery that mTORC1 regulates biogenesis of miRNA further expands the repertoire of this master regulator for mammalian cellular and developmental processes.

There are two distinct chromosomal loci for the miR-1 gene, miR-1-1 and miR-1-2. An upstream enhancer has been found at each locus to mediate the regulation of miR-1 expression in cardiac muscle, and regulatory sites for serum response factor, MyoD and MEF2, have been identified in the enhancers (Zhao et al., 2005). Our results have indicated that the miR-1 enhancer activity is indeed up-regulated during skeletal myocyte differentiation and inhibited by rapamycin, suggesting mTORC1 regulation of miR-1 expression through this enhancer. We have further shown that MyoD mediates mTOR regulation of the miR-1 enhancer activity and that mTORC1 controls the protein stability of MyoD. mTOR regulation of MyoD and miR-1 is most likely direct, based on the following observations: (a) the effects of rapamycin on MyoD degradation and miR-1 enhancer activity were acute and not dependent on myogenic...
miRNA profiling of differentiating C2C12 cells has been reported by other groups (Chen et al., 2006; Wong and Tellam, 2008). Although there is some degree of agreement within the results obtained by different groups including ours, our data have revealed more myogenically regulated miRNAs than the other studies, possibly because of the difference in the miRNA chips used, robustness of cell differentiation, and/or methods of data analysis (e.g., we used statistical significance rather than fold change to set the threshold for data selection). In any event, our array results have confirmed the up-regulation during differentiation of almost all reported regulators of myogenesis in addition to miR-1, including miR-24, -26a, -27b, -133, -206, -181, -214, and -499 (Table S1; Naguibneva et al., 2006b; Sun et al., 2008; Crist et al., 2009; Juan et al., 2009; van Rooij et al., 2009; Williams et al., 2009). Many miRNAs not previously reported to be involved in myogenesis are found to be up- or down-regulated during differentiation (Table S1), potentially representing novel regulators of myogenic pathways that are worthy of future investigation. Intriguingly, among ~50 miRNAs that were differentially expressed during myogenesis (out of ~500 total), 24 miRNAs other than miR-1 displayed various degrees of rapamycin sensitivity (Fig. S1 and Table S1). Although some of these miRNAs may lie far downstream of mTORC1 in myogenesis, it is conceivable that other miRNAs in addition to miR-1 may be directly regulated.

differentiation (Fig. 4, D–F), and (b) although rapamycin abolished miR-1 enhancer activity, other drugs that block differentiation did not have any effect (Fig. 3). In addition to the upstream enhancers of miR-1 genes, an intragenic enhancer has been reported to activate muscle-specific transcription of the bicistronic primary transcript encoding miR-1-2 and miR-133a-1 (Liu et al., 2007). Interestingly, we found that a reporter of this intragenic enhancer was activated during C2C12 cells differentiation and inhibited by rapamycin (Fig. S5). Because MyoD also regulates this enhancer (Liu et al., 2007), it raises the possibility that the mTORC1–MyoD axis controls miR-1 transcription at multiple levels.

In contrast to mTOR regulation of ribosomal DNA transcription, which requires S6K1 (Mayer et al., 2004), mTOR regulation of miR-1 expression does not seem to involve S6K1, as knockdown of S6K1 has no effect on miR-1 expression during myogenesis (unpublished data). It is conceivable that mTORC1 phosphorylates MyoD and, in turn, stabilizes MyoD. We have indeed observed that mTOR phosphorylates MyoD in vitro (unpublished data). The reported MyoD phosphorylation sites, Ser5 and Ser200, do not conform to this model, as their phosphorylation correlates with degradation of MyoD (Song et al., 1998; Tintignac et al., 2004). The exact mechanism by which mTORC1 regulates MyoD stability is currently under investigation.
by mTORC1. Further characterization of those candidate miRNAs will likely enhance our understanding of the myogenic regulatory network.

**Follistatin as an mTOR-regulated fusion factor**

Previously, we reported the existence of a fusion factor under the control of mTORC1 signaling in an mTOR kinase-dependent manner, which promoted maturation of myotubes arrested at the nascent myotube stage by the treatment of rapamycin (Park and Chen, 2005). Our current study has revealed follistatin as this long-sought fusion factor regulated by mTORC1 through an miR-1-mediated pathway. Several other secreted factors have been found in the conditioned medium of normally maturing myotubes but not of rapamycin-arrested nascent myotubes (unpublished data), including the established second-stage fusion factor IL-4 (Horsley et al., 2003) and the NF-kB-induced myogenic stimulator IL-6 (Baeza-Raja and Muñoz-Cánoves, 2004). However, none of them are able to rescue rapamycin-inhibited myotube maturation (unpublished data). The capacity of exogenous follistatin to fully rescue from rapamycin inhibition myotube maturation in vitro (Fig. 9) and muscle regeneration in vivo (Fig. 10, B and C), together with the inhibitory effect of rapamycin on follistatin expression both in vitro and in vivo (Fig. 8 B and Fig. 10 A), makes follistatin the major, if not the only, fusion factor regulated by mTORC1.

Follistatin, an activin-binding protein essential for multiple aspects of mouse development (Matzuk et al., 1995), is thought to control skeletal muscle development through antagonizing the myogenic inhibitor myostatin (Lee and McPherron, 2001; Amthor et al., 2004). The stimulatory effect of follistatin on adult muscle growth has been demonstrated in the mdx mice (Minetti et al., 2006; Haidet et al., 2008; Nakatani et al., 2008). In vitro, follistatin was shown to mediate TSA- or nitric oxide/cyclic GMP–induced myoblast fusion (Iezzi et al., 2004; Pisconti et al., 2006). It is likely that follistatin plays a role in both the initial myoblast fusion and the second-stage fusion, as we have observed that differentiating C2C12 cells exposed to recombinant follistatin display increased fusion index in addition to increased size of myotubes (unpublished data). This well corroborates our observation that inhibiting miR-1 results in decreased myotube size and fusion index (Fig. 6), supporting the notion that miR-1 and follistatin act on the same pathway to regulate two stages of myocyte fusion. The new regulatory pathway for follistatin (Fig. 10 D) discovered in our study should expand the therapeutic potential of this important myogenic factor.

**Materials and methods**

**Antibodies and other reagents**

The antibodies were obtained from the following sources: anti-tubulin was obtained from Abcam, anti-MyoD (5.BA) was obtained from Imgenex, antibodies against HDAC4, mTOR, phospho-T389-S6K1, MEF2A, and MEF2C were obtained from Cell Signaling Technology, and all secondary antibodies and DAPI were obtained from Jackson ImmunoResearch Laboratories, Inc. The MF20 anti-sarcomeric MHC and F5D anti-myogenin antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development, the National Institutes of Health, and maintained by
The University of Iowa Department of Biological Sciences. Rapamycin was obtained from LC Laboratories. Follistatin (mouse FS288) was obtained from R&D Systems. TSA, gelatin, polybrene, puromycin, MG-132, and custom-designed LNA oligonucleotides were obtained from Sigma-Aldrich.

**Tissue culture**

C2C12 and C3H10T1/2 cells were maintained in DME (1 g/liter glucose) with 10% fetal bovine serum. For differentiation, cells were seeded in plates coated with 0.2% gelatin. Differentiation was induced by switching to DME containing 2% horse serum. Transfection of LNA anti-miR-1 and luciferase reporters into C2C12 cells was performed using nucleofector (program V; solution B-032; Lonza) following the manufacturer’s recommendations. All other transfections were performed with Trans-IT (Mirus) following the manufacturer's protocols. cDNA was synthesized from total RNA using the mirVana RNA isolation kit (Applied Biosystems). cDNA was synthesized using the SuperScript II; Invitrogen. Reverse transcription was performed using the high-capacity cDNA retrotranscription kit with random primers (Applied Biosystems) following the manufacturer’s protocols. 20 µg/sample RNA was labeled with [γ-32P]ATP. The RNA Decade Maker system (Applied Biosystems) was similarly radiolabeled as size markers. 20 µg/sample RNA was separated on 1% denaturing polyacrylamide gels, transferred to nylon membranes (GE Healthcare), and UV cross-linked. Prehybridization was performed in ultrahyb hybridization buffer (Ultradyn; Applied Biosystems) for 2 h at 42°C followed by hybridization with probe in the same buffer (10°C CPM/ml) overnight at 42°C. The membranes were washed with 2× SSC and 0.1% SDS at 42°C followed by exposure to x-ray films overnight at –80°C.

**Microarray analysis**

miRNA profiling was performed using LNA miRNA arrays (Exiqon), which were purchased from Sigma-Aldrich. miRBase (http://www.mirbase.org/). Samples were prepared using miRVana miRNA isolation kit (Applied Biosystems) without enrichment for small RNA. 1 µg total RNA was labeled with Cy3 or Cy5 according to the manufacturer’s protocols cohybridized to arrays overnight, washed, and scanned with a fluorescence scanner (GenePix 4000B; MDS Analytical Technologies). Image analysis and editing were performed using GenePix Pro (version 6.0). Data were analyzed using the bioconductor packages with R software. For each comparison, false discovery rate–adjusted p-value at 0.05 was used as cutoff for statistical significance.

**Muscle regeneration in mice**

All animal experiments in this study followed protocols approved by the Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. 10–12-wk-old male FVB mice were used in all experiments. Muscle injury was induced by injection of 50 µl BaCl2 (1.2% wt/vol) into the TA muscle of the hind limb. As control, saline was injected into the TA muscle of the hind limb. To induce muscle regeneration, muscle cryosections were fixed in paraformaldehyde, deproteinized with a Fluotar 20× 0.4 NA dry objective (Leica) using a camera (RETIGA Exi; QImaging). The images were processed as 24-bit colored images using Photoshop (CS2; Adobe). MHC and DAPI signals were pseudo-colored green and blue, respectively. The fusion index was calculated as the ratio of nuclei number in myocytes with two or more nuclei versus the total number of nuclei. Each data point was generated from at least 200 randomly chosen MHC-positive cells or myotubes.

**ELISA for follistatin measurement**

Relative follistatin protein levels in media collected from differentiating C2C12 cells were measured using the human follistatin ELISA kit (26% cross reactivity with mouse follistatin; R&D Systems) following the manufacturer’s protocols.

**Northern blotting**

Antisense DNA oligonucleotides for mature miR-1 and let-7a were labeled with γ-[32P]ATP. The RNA Decade Maker system (Applied Biosystems) was similarly radiolabeled as size markers. 20 µg/sample RNA was separated on 1% denaturing polyacrylamide gels, transferred to nylon membranes (GE Healthcare), and UV cross-linked. Prehybridization was performed in ultrahyb hybridization buffer (Ultradyn; Applied Biosystems) for 2 h at 42°C followed by hybridization with probe in the same buffer (10°C CPM/ml) overnight at 42°C. The membranes were washed with 2× SSC and 0.1% SDS at 42°C followed by exposure to x-ray films overnight at –80°C.

**Luciferase assays**

Cells transfected with various enhancer reporters were treated as described in Results and Figs. 3 and 4 and lysed in Passive Lysis buffer (Promega). Luciferase assays were performed using the Luciferase Assay Systems kit (Promega) following the manufacturer’s protocol.

**Immunofluorescence microscopy and quantitative analysis of myocytes**

C2C12 cells differentiated in 12-well plates were fixed in 3.7% formaldehyde (in PBS), permeabilized in 0.1% Triton X-100, and incubated with fluorescein isothiocyanate-conjugated anti–mouse IgG in 3% BSA (in PBS) followed by fluorescein isothiocyanate-conjugated anti–mouse IgG in 3% BSA (in PBS) with 4 µg/ml DAPI. The stained cells were examined with a fluorescence microscope (DMi 4000B; Leica), and the fluorescent images were captured using a camera (RETIGA Exi; QImaging). The images were processed as 24-bit images using Photoshop (CS2; Adobe). MHC and DAPI signals were pseudo-colored green and blue, respectively. The fusion index was calculated as the ratio of nuclei number in myocytes with two or more nuclei versus the total number of nuclei. Each data point was generated from at least 200 randomly chosen MHC-positive cells or myotubes.

** Luciferase assay**

Cells transfected with various enhancer reporters were treated as described in Results and Figs. 3 and 4 and lysed in Passive Lysis buffer (Promega). Luciferase assays were performed using the Luciferase Assay Systems kit (Promega) following the manufacturer's protocol.

**Lentivirus-mediated RNAi**

shRNAs in the pLKO.1-puro vector for knocking down mTOR and HDAC4 were purchased from Sigma-Aldrich (MISSION TRC). For viral packaging, pLKO-shRNA, pCMV-dR8.91, and pCMV–VSV-G were cotransfected into 293 cells and purified by ultracentrifugation with a cesium chloride gradient (Luo et al., 2007) followed by dialysis against PBS. The viruses were amplified through infection of 293 cells and purified by ultracentrifugation with a cesium chloride gradient (Luo et al., 2007). miRNAs were quantified using quantitative PCR–based miRNA assay kits (Taqman; Applied Biosystems) following the manufacturer’s recommendations, with snRNA 202 as the internal control for normalization.

**Real-time quantitative PCR for miRNA and mRNA**

Total RNA was isolated from cultured cells or muscle tissues using the mirVana RNA isolation kit (Applied Biosystems). cDNA was synthesized from 2 µg total RNA with reverse transcription (SuperScript II; Invitrogen) using oligo (dT) primer (Invitrogen). Quantitative PCR was performed on an iCycler system (Bio-Rad Laboratories) using a SYBR green PCR kit (Applied Biosystems) in a 96-well reaction plate (MicroAmp; Bio-Rad Laboratories) following the manufacturer’s protocols. β-Actin was used as a reference to obtain the relative fold change for target samples using the comparative C method. The following primers were used: mouse follistatin (forward), 5′-AAAACTCCACCAGGAGT-3′ and (reverse) 5′-GGCTGATCCAC-CACAGAG-3′; and mouse β-actin (forward), 5′-TTCGCAAGGATG-CAGAAG-3′ and (reverse) 5′-ATCCACATCCTGCTGAAAG-3′. Primers for pri-miR-1-1 and pri-miR-1-2 were used as described previously (Liu et al., 2007). miRNAs were quantified using quantitative PCR–based miRNA assay kits (Taqman; Applied Biosystems) following the manufacturer’s recommendations, with snRNA 202 as the internal control for normalization.

**Muscle tissue cryosection and histological analysis**

TA muscles were isolated by dissection, frozen in liquid nitrogen–cooled 2-methylbutane, and embedded in TBS tissue freezing medium (Thermo Fisher Scientific). Sections of 10-µm thickness were made with a cryostat (Micron HM550; Thermo Fisher Scientific) at –20°C, placed on uncoated slides, and stained with hematoxylin and eosin. The stained slides were examined with a microscope (DMi 4000B), and the images were captured with a Fluotar 20× 0.4 NA dry objective (Leica) using a camera (RETIGA Exi; QImaging). The images were processed as 24-bit images using Photoshop (CS2; Adobe). MHC and DAPI signals were pseudo-colored green and blue, respectively. The fusion index was calculated as the ratio of nuclei number in myocytes with two or more nuclei versus the total number of nuclei. Each data point was generated from at least 200 randomly chosen MHC-positive cells or myotubes.

**In situ hybridization**

In situ hybridization of miR-1 and let-7a was performed on cryosections of TA muscles prepared as described in the previous paragraph. miR-1 was detected by antisense LNA probes labeled with the Dig-3 end-labeling kit (Roche) as previously described (Nagirneva et al., 2006a) with some modifications. In brief, muscle cryosections were fixed in paraformaldehyde, deproteinized...
with proteinase K, and acetylated with acetic anhydride and triehanol-amine followed by incubation with 20 nM labeled probes in hybridization buffer (65% formamide, 5x SSC, 0.1% Tween 20, 50 mg/ml heparin, and 500 mg/ml yeast RNA, pH 6.0) at 50°C overnight. The slides were subsequently washed with 50% formamide/2x SSC at 50°C and with PBST at room temperature followed by blocking and incubation with anti-digoxigenin (1:1,000, Roche) at 4°C overnight. The slides were washed and developed in NBT/BCIP [Sigma-Aldrich] for 4 h, dehydrated, and mounted. A probe with a scrambled sequence was used as a negative control. The following probes were used [bold letters indicate LNA]: anti–miR-1 LNA probe, 5'-ATACACATCTTACTCAACCTCAAC-3'; and scrambled LNA probe, 5'-CATGATGTTGCACTCTTC-3'. An anti-miR-1 probe purchased from Exiqon was also used, and similar in situ hybridization signal patterns were observed.

### Statistical analysis

All data are presented as mean ± SD. Whenever necessary, statistical significance of the data was analyzed by performing one-sample t tests, paired two-tailed t tests, or one-way analysis of variance with Bonferroni’s postest using Prism (version 4.0; GraphPad Software, Inc.). The specific types of tests and the p-values, when applicable, are indicated in Figs. 5–8 and 10.

### Online supplemental material

Fig. S1 and Table S1 show the results of miRNA profiling in differentiating C2C12 cells. Fig. S2 shows rapamycin inhibition of myogenic markers. Fig. S3 shows in situ hybridization of miR-1 in regenerating muscle. Fig. S4 shows MyoD mRNA levels in C2C12 and C3H10T1/2 cells upon rapamycin treatment. Fig. S5 shows rapamycin inhibition of the miR-1/133a reporter and follistatin-expressing adenovirus.

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## References


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